THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 23, pp. 17148–17156, June 8, 2007

Epitope Analysis of the Malaria Surface Antigen Pfs48/45 Identifies a Subdomain That Elicits Transmission Blocking Antibodies*

Received for publication, February 1, 2007, and in revised form, April 9, 2007 Published, JBC Papers in Press, April 9, 2007, DOI 10.1074/jbc.M700948200

Nikolay Outchkourov[‡1], Adriaan Vermunt[‡1], Josephine Jansen[‡], Anita Kaan[‡], Will Roeffen[§], Karina Teelen[§], Edwin Lasonder[‡], Anneke Braks[‡], Marga van de Vegte-Bolmer[§], Li Yan Qiu[‡], Robert Sauerwein[§], and Hendrik G. Stunnenberg[‡2]

From the [‡]Department of Molecular Biology, NCMLS, Radboud University Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands; [§]Department of Medical Microbiology, Radboud University Nijmegen Medical Center, 268, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Pfs48/45, a member of a *Plasmodium*-specific protein family, displays conformation-dependent epitopes and is an important target for malaria transmission-blocking (TB) immunity. To design a recombinant Pfs48/45-based TB vaccine, we analyzed the conformational TB epitopes of Pfs48/45. The Pfs48/45 protein was found to consist of a C-terminal six-cysteine module recognized by anti-epitope I antibodies, a middle four-cysteine module recognized by anti-epitopes IIb and III, and an N-terminal module recognized by anti-epitope V antibodies. Refolding assays identified that a fragment of 10 cysteines (10C), comprising the middle four-cysteine and the C-terminal six-cysteine modules, possesses superior refolding capacity. The refolded and partially purified 10C conformer elicited antibodies in mice that targeted at least two of the TB epitopes (I and III). The induced antibodies could block the fertilization of *Plasmodium falciparum* gametes *in vivo* in a concentration-dependent manner. Our results provide important insight into the structural organization of the Pfs48/45 protein and experimental support for a Pfs48/45-based subunit vaccine.

Malaria parasites are spread in the human population by *Plasmodium*-infected Anopheles mosquitoes. After a blood meal on infected humans, mosquitoes become infected by ingesting a sexual form of the malaria parasite called gametocytes. Subsequent sporogonic development in the mosquito can be prevented by the presence of anti-malaria, transmission-blocking (TB)[3] antibodies in the ingested blood meal (1–3). Pfs48/45 is a TB vaccine candidate that belongs to a family of malaria-specific proteins that contain conserved motifs with four- or six-cysteine residues (4). Pfs48/45 plays a key role in parasite fertilization (5), and monoclonal antibodies (mAbs) against Pfs48/45 prevent fertilization (6). Anti-Pfs48/45 antibodies are present in human sera from endemic areas, and there is evidence that the presence of anti-Pfs48/45 antibodies in natural sera correlates with TB activity (7). The induction of antibodies after natural infection, as observed in the field, creates the highly beneficial potential of vaccine boosting in the endemic setting. At least four different epitopes (I, IIb, III, and V) on Pfs48/45 are targeted by monoclonal antibodies that block or reduce malaria transmission (8, 9). Apart from epitope V, the TB epitopes are sensitive to reducing agents, which indicates the importance of the disulfide bridges and hence the conformational dependence of these epitopes (8, 10).

To design a Pfs48/45-based TB vaccine, we aimed to delineate and characterize the TB epitopes of Pfs48/45. Protease digestion of the native protein, expression in *Escherichia coli* of truncations, cysteine mutations, and refolding assays were evaluated by immunological analysis of the TB epitopes. An N-terminally truncated protein with higher refolding efficiency was identified, partially purified, and tested in mice immunization experiments. The elicited antibodies recognized the native Pfs48/45 protein, competed with the TB mAbs against epitopes I and III, and displayed TB activity. Our study provides the first biochemical analysis of the Pfs48/45 protein and a blueprint for the design of Pfs48/45-based vaccines.

EXPERIMENTAL PROCEDURES

Native Pfs48/45 Protein—Mature *Plasmodium falciparum* (NF54) gametocytes were produced as previously described (11) and extracted in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Insoluble debris was removed by centrifugation at 13,000 × *g* for 5 min at room temperature. The supernatant contained native Pfs48/45 protein that was used in Western blots and enzyme-linked immunosorbent assays (ELISAs).

Antibodies—Polyclonal rabbit antiserum K96 was used to detect recombinant Pfs48/45 (12). To detect epitopes of Pfs48/45, various mAbs from mouse origin, 32F5 (epitope I) (6) or from rat origin, 85RF45.1 (epitope I), 85RF45.2b (epitope IIb), 85RF45.3 (epitope III), and 85RF45.5 (epitope V) (8) were used.

* This study was financially supported by European Malaria Vaccine Initiative Grant 041222 and by EuroMalvac II Contract QLK2-CT-2002-01197. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These authors contributed equally to the presented work.

[2] To whom correspondence should be addressed: Dept. of Molecular Biology, NCMLS M850/3.79, Radboud University, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel.: 31-24-3610524; Fax: 31-24-3610520; E-mail: h.stunnenberg@ncmls.ru.nl.

[3] The abbreviations used are: TB, transmission-blocking; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; LC-MS/MS, liquid chromatography/tandem mass spectrometry; GST, glutathione *S*-transferase; Ni-NTA, nickel-nitrilotriacetic acid; IFA, immunofluorescence assay; SIFA, surface IFA.

Alkaline phosphatase-conjugated antisera were purchased from Sigma and horseradish peroxidase (HRP)-conjugated antisera from Nordic.

Partial Protease Digestion and Purification of Pfs48/45 Protease Fragment—Partial protease treatment was conducted with trypsin (sequencing grade, Roche Applied Science). For each digestion, 5×10^5 gametocyte equivalents of the above-described deoxycholate extract were used. The proteins were digested at 25 °C for 1 h. The amount of protease varied from 0.01, 0.1 to 1 μg in a final reaction volume of 10 μl. The digestion was stopped by adding a protease inhibitor mixture (Complete™, Roche Applied Science). For preparative immunopurification of protease fragments, 1×10^8 gametocyte equivalents were digested with 1 μg/ml trypsin for 2.5 h at 30 °C. A 28-kDa fragment was purified using mAb 32F5 against Pfs48/45 epitope I. This mAb 32F5 was cross-linked to protein G-Sepharose 4B Fast Flow (Sigma) using dimethylpimelimidate (Sigma) (13). The trypsin-treated extract was incubated overnight at 4 °C with the cross-linked beads and washed three times with 0.1% deoxycholate in phosphate-buffered saline (PBS) and three times with RIPA buffer as described in Ref. 13. Bound proteins were eluted twice with 500 μl of 1% SDS in PBS at 70 °C (14). To identify the 28-kDa fragment, the eluted proteins were SDS-PAGE-separated and stained with Coomassie Blue R-250 (Sigma). The 28-kDa doublet was sliced out from the gel and analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS), as previously described (15).

Constructs of Truncated and Mutated Pfs48/45—The coding sequence of Pfs48/45 (14) without the predicted signal peptide and glycosylphosphatidylinositol signal sequence (amino acids 27–427) was cloned into pGEX-2T (Amersham Biosciences), resulting in an N-terminal glutathione *S*-transferase (GST) fusion. N-terminally truncated Pfs48/45 GST fusions were generated using standard cloning techniques. Cysteines were mutated to serines by site-directed mutagenesis (QuikChange, Stratagene). For refolding experiments, Pfs48/45 fragments were cloned in the vector pET-15b (Novagen), resulting in N-terminally His_6-tagged proteins. All constructs were verified by sequencing using BigDye (Applied Biosystems, ABI) and the ABI PRISM 310 genetic analyzer.

Purification and Refolding of E. coli-expressed Proteins—The tagged proteins (GST or His) were expressed in the *E. coli* strain BL21 DE3 (Novagen) by induction for 3 h at 37 °C with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (GST fusions) or 1 mM isopropyl 1-thio-β-D-galactopyranoside (His-tagged) at an A_{600nm} of 0.6. The expressed proteins were analyzed in crude lysates or after affinity purification. Bacteria were lysed by ultrasonic homogenization in PBS, 1 mg/ml lysozyme, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Complete™, Roche Applied Science). After centrifugation (10,000 × *g* for 15 min at 4 °C), pellets containing the inclusion bodies were washed with 0.5% Triton X-100 in 10 mM Tris, pH 8.0, and 1 mM EDTA followed by 0.5% Triton X-100 in 10 mM Tris, pH 8.0. The His-tagged proteins were purified using Ni-NTA beads (Qiagen) after solubilization of the inclusion bodies in 8 M urea, 50 mM Tris-HCl, pH 8, 0.5 M NaCl, and 1 mM β-mercaptoethanol. Following centrifugation (10 min 10,000 × *g*), the supernatant was loaded on a 10-ml disposable column (Bio-Rad) with a 400-μl bed volume of NTA beads. After 30 min of incubation at room temperature during slow vertical rotation, the beads were allowed to settle and the column was washed with 10 bed volumes of solubilization buffer and 10 bed volumes of 8 M urea, 50 mM Tris-HCl, pH 8, and 1 mM reduced glutathione. The tagged proteins were eluted two times with 0.75 ml of 10 mM EDTA in 8 M urea, 0.5 M NaCl, 50 mM Tris-HCl, pH 8.0, and 1 mM glutathione. The eluted proteins at a concentration of 0.25 μg/μl were 8-fold dropwise-diluted in renaturation buffer (PBS, 0.1% Triton X-100, 1 mM reduced glutathione, and 1× protease inhibitor mixture (Complete™, Roche Applied Science) and refolded overnight at 4 °C.

Refolding on Column and Purification of 10C Fragment—Inclusion bodies were prepared and solubilized as described above. The protein was then bound to a Ni-NTA resin, and the column was washed with 8 M urea, 50 mM Tris-HCl, pH 8, 0.5 M NaCl. The protein was refolded by subsequent buffer exchange to PBS that contained 0.1% Triton X-100, and the bound, refolded protein was eluted with 10 mM EDTA in the above-described buffer. The eluted proteins were 50-fold-concentrated on Vivaspin 20, 30,000 molecular weight cutoff filters (Vivascience). Insoluble aggregates were removed by centrifugation (30 min at 13,000 × *g*), and protein was further purified over a Superdex 75 HR 10/30 column (Amersham Biosciences).

SDS-PAGE and Immunoblot Analysis—Protein samples were mixed with an equal amount of SDS-PAGE loading buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 20% glycerol, and 0.02% bromphenol blue) and, unless otherwise indicated, boiled for 5 min at 95 °C without any reducing agent. The samples were resolved on 10% SDS-PAGE gels and visualized by Coomassie Blue R-250 staining. The presence of Pfs48/45 and epitope recognition was analyzed by immunoblot analysis using nitrocellulose filters (Protran, Schleicher & Schuell). The electrophoretic transfer was carried out for 2 h at 50 V, 100 mA. The membranes were blocked overnight at 4 °C in 4% milk (Elk, Campina, The Netherlands) in PBS with 0.2% Tween 20 (PBST). Incubations with primary (1.5 h) and secondary mAbs (1 h) were performed in blocking buffer. The filters were washed three times for 5 min with PBST in between antibody incubations. ECL substrate (Amersham Biosciences) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (13) were used for detection.

Animal Immunizations—Groups of female BALB/c mice (6 weeks old, five mice/group) were immunized subcutaneously with recombinant 10C fragment or parasites in a total volume of 0.1 ml emulsified in complete Freund's adjuvant, and boosts were given after 28, 42, and 133 days using incomplete Freund's adjuvant. Group 1 was immunized with 25 μg of recombinant 10C per mouse, group 2 with 10^6 NF54 parasites, group 3 with 10^5 NF54 parasites, and group 4 was immunized with adjuvant alone. Serum was collected on days 0, 14, 42, 56, and 147 and tested for specific antibody reactivity.

Immunofluorescence Assay (IFA)—An indirect IFA was performed with a mix of cultured asexual and sexual stage parasites (NF54 isolate of *P. falciparum*) air-dried on multispot slide, as described previously (8, 11). Briefly, parasites were incubated with a 1:100 dilution of the test sera in PBS, rinsed with PBS, and

incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (MP Biomedicals). The slides were rinsed, washed, mounted under a coverslip, and examined under ultraviolet illumination with a Leitz Ortholux fluorescence microscope (500× magnification). For surface IFA (SIFA) analysis, gametocytes were allowed to undergo gametogenesis, as described previously (8, 11). Subsequently, suspension of macrogametes/zygotes (100 μl) was mixed with 100 μl of a 1:100 dilution of the sera in PBS and incubated for 20 min on ice. Parasites were washed with 1 ml of PBS, collected by centrifugation for 3 min at 3000 revolutions/min (benchtop centrifuge), and incubated with 50 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (MP Biomedicals). After a wash with PBS, the cells were examined as described for the IFA.

Direct ELISA—ELISA plates were coated overnight with gametocyte extract (250,000 parasites/well) in PBS. After blocking with 5% milk in PBS, sera (in the desired dilutions) were incubated for 2 h. Bound antibodies were detected by HRP-conjugated goat anti-Mouse IgG and visualized using 3,3′,5,5′-tetramethyl benzidine substrate, as previously described (8, 11).

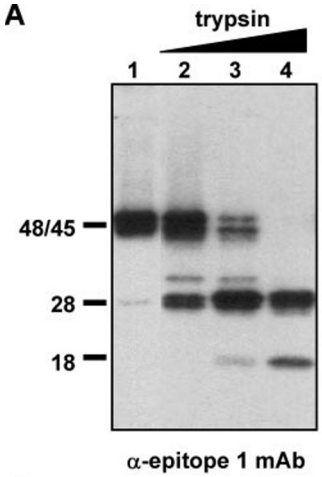
Pfs48/45-specific ELISA—A two-sided ELISA was performed as previously described (8, 11). Samples were treated with 10 mM iodoacetamide just before the serial dilution to prevent refolding in the wells. The amount of the native Pfs48/45 protein in the gametocyte extract was predetermined by Western blot analysis under reducing conditions, using the polyclonal antiserum K96 (12) and the recombinant protein as a reference.

Competition ELISA—The Pfs48/45 protein present in gametocyte extract was captured essentially as for the Pfs48/45-specific ELISA using mAb 85RF45.2b. Competition of HRP-labeled 85RF45.1 or 85RF.45.3 with sera antibodies from 10C-immunized mice was performed by incubation of 30 μl of HRP-labeled mAb (2.5 μg/ml, diluted with PBS containing 0.1% milk) and 30 μl of mice serum (dilutions ranging from 1:20 to 1:2560 diluted with PBS containing 0.1% milk) for 2 h. Bound, labeled mAbs were measured as described above.

Standard Membrane-feeding Assay—Antisera obtained from mice immunized with the 10C fragment were compared for their TB activities in standard membrane-feeding assays, as previously described (16, 17). Briefly, 30 μl of the mice sera were mixed with 60 μl of naïve human serum and 180 μl of *in vitro* gametocyte culture of *P. falciparum* (NF54 line). This mixture was fed to *Anopheles stephensi* mosquitoes (Nijmegen strain) through a membrane-feeding apparatus. Sera from mice immunized with PBS buffer alone served as the controls. Blood-fed mosquitoes were kept at 26 °C and 80% humidity for 7 days. Surviving mosquitoes (>90%) were dissected and oocysts counted from extracted midguts. Twenty mosquitoes/batch were examined for the presence of oocysts. A standard membrane-feeding assay experiment was considered valid when, in three control batches of mosquitoes fed on naïve human serum and *in vitro* gametocyte culture, the percentage of infected mosquitoes was 90% or more.

RESULTS

Protease Mapping of the Pfs48/45—To elucidate the domain organization of the Pfs48/45 protein, trypsin digestion was used.



B

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  1 MMLYISAKKA QVAFILYIVL VLRIISGNND FCKPSSLNSE ISGFIGYKCN
 51 FSNEGVHNLK PDMRERRSIF CTIHSYFIYD KIRLIIPKKS SSPEFKILPE
101 KCFQKVYTDY ENRVETDISE LGLIEYEIEE NDTNPNYNER TITISPFSPK
151 DIEFFCFCDN TEKVISSIEG RSAMVHVRVL KYPHNILFTN LTNDLFTYLP
201 KTYNESNFVS NVLEVELNDG ELFVLACELI NKKCFQEGKE KALYKSNKII
251 YHKNLTIFKA PFYVTSKDVN TECTCKFKNN NYKIVLKPKY EKKVIHGCNF
301 SSNVSSKHTF TDSLDISLVD DSAHISCNVH LSEPKYNHLV GLNCPGDIIP
351 DCFFQVYQPE SEELEPSNIV YLDSQINIGD IEYYEDAEGD DKIKLFGIVG
401 SIPKTTSFTC ICKKDKKSAY MTVTIDSAYY GFLAKTFIFL IVAILLYI
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FIGURE 1. **Trypsin digestion of the native Pfs48/45.** *A*, gametocyte extract (5×10^5 gametocyte equivalents) was treated with increasing amounts of trypsin for 1 h at 25 °C. *Lanes 1–4*, 0.01, 0.1 to 1 μg of trypsin. The samples were resolved by non-reducing SDS-PAGE and immunoprobed with anti-epitope I mAb. *B*, Pfs48/45 amino acid sequence. LC-MS/MS-identified peptides of the 28-kDa fragment are *underlined*.

Trypsin recognition sites that are buried inside the protein should be relatively inaccessible. The Pfs48/45 protein contains, in total, 51 Lys and Arg residues, all of which are potential trypsin cleavage sites. Fig. 1 shows the result of a trypsin digestion on gametocyte extract separated under non-reducing conditions on SDS-PAGE and analyzed by immunoblot with anti-Pfs48/45 epitope I mAb. A doublet migrating at ~28 kDa reacted with TB epitope I (Fig. 1*A*) as well as with IIb and III mAbs but not with epitope V mAb (data not shown). Immunopurification of the 28-kDa doublet using epitope I mAb and subsequent LC-MS/MS analysis identified peptides between residues 164 and 404 with a corresponding molecular weight of 28 kDa. Thus, Pfs48/45 contains a C-terminal part of the 28-kDa that is relatively resistant to trypsin cleavage and that displays conformational epitopes I, IIb, and III. At higher trypsin concentrations, a minor 18-kDa fragment was generated with weak epitope I (Fig. 1*B*) but with no epitopes IIb and III reactivity (data not shown).

Expression of GST-fused N-terminal Pfs48/45 Truncations in E. coli—To determine more precisely which regions are critical for epitope formation, full-length Pfs48/45 without its N-terminal signal peptide sequence and its C-terminal signal for attachment of a glycosylphosphatidylinositol moiety (amino acids 27–427), the 16C fragment (Fig. 2*A*), and truncations thereof were constructed and expressed as N-terminal GST fusions in *E. coli*. Total *E. coli* proteins were boiled in SDS sample buffer with 50 mM dithiothreitol, resolved by SDS-PAGE, and blotted onto nitrocellulose membranes. The observed reactivity with the mAbs after blotting is likely a result of refolding of the pro-

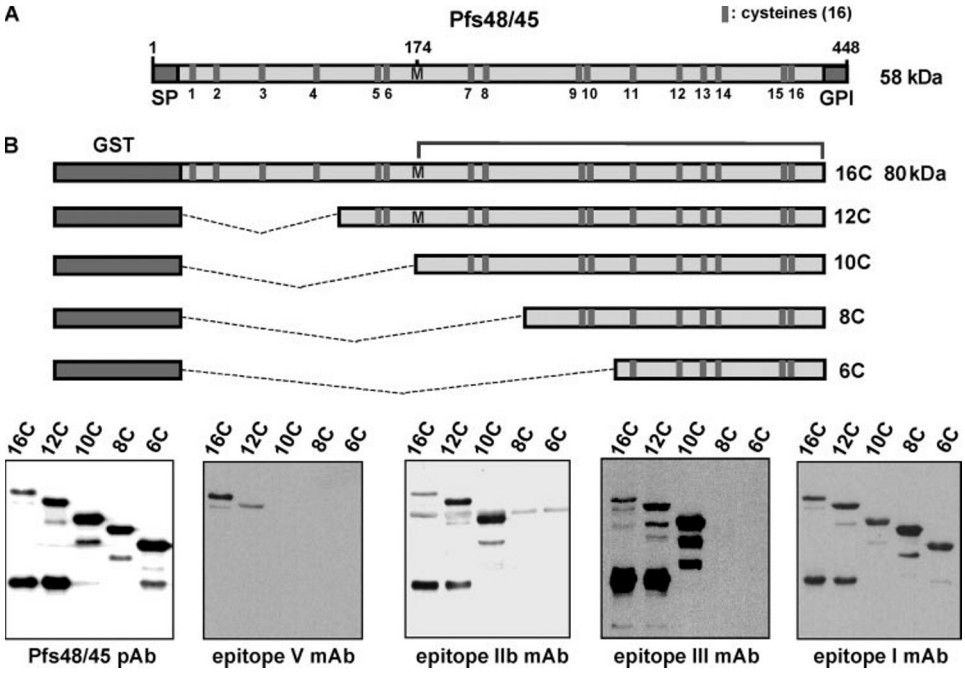

FIGURE 2. **Effects of N-terminal truncations on the Pfs48/45 TB epitopes.** *A*, a schematic representation of the Pfs48/45 protein. The coding sequence contains a total of 448 amino acids and includes an N-terminal signal peptide (*SP*) and a C-terminal signal for attachment of a glycosylphosphatidylinositol (*GPI*) moiety. *Bars* indicate the relative position of cysteine residues. Methionine (*M*) at position 174 is indicated on *top* as a most likely internal translation initiation product. *B*, overview of N-terminal truncations. Shown are 16C (residues 27–427), 12C (108–427), 10C (173–427), 8C (236–427), and 6C (295–427), which are expressed in *E. coli* as fusions with GST. Crude *E. coli* extracts were screened with mAbs against epitopes I, IIb, III, and V and anti-Pfs48/45 polyclonal sera K96. Samples were boiled in SDS sample buffer with 50 mM dithiothreitol prior to loading on SDS-polyacrylamide gels. The observed reactivity is an expected result of refolding on the blot. *Asterisks* indicate the position of the internal translation initiation product.

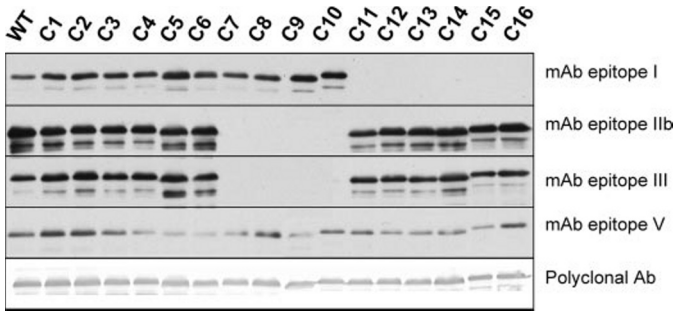

FIGURE 3. **Effects of cysteine mutations on the Pfs48/45 TB epitopes.** Individual cysteine to serine substitutions (Cys-1–Cys-16) were introduced in the GST-16C construct (see Fig. 2*B*). Upon expression in *E. coli* and refolding on the nitrocellulose membrane, the recognition of by mAbs was analyzed by Western blots.

tein on the nitrocellulose membrane. Expression of full-length Pfs48/45 fused to GST (Fig. 2*B*, *16C*) resulted in a protein of ~80 kDa that was recognized by the polyclonal, non-conformation-dependent K96 sera (12) and by the mAbs recognizing epitopes I, IIb, III, and V (8) (Fig. 2*B*). A second polypeptide with an apparent molecular weight of 30 kDa was detected, which was generated by the initiation of translation at an internal start codon at methionine 174 due to the presence of a Shine-Delgano-like motif. This polypeptide was recognized by mAbs I, IIb, and III and was absent when using constructs shorter than 10C; it migrated at the same position as 10C without the GST moiety.

In line with the trypsin sensitivity assays, the 10C fragment could be detected by epitopes I, IIb, and III mAbs. Furthermore, epitopes IIb and III were present in the 10C but not in the 8C or shorter fragments. Epitope I was present in all of the C-terminal truncations, including the 6C fragment (Fig. 2*B*). The mAb against epitope V displayed a much stronger reactivity with the 16C than the 12C construct, and no reactivity could be detected against the 10C or shorter fragments. Thus, the region between cysteines 4 and 7 appeared to be essential for epitope V, and the region N-terminal to cysteine 4 appeared to contribute to epitope V formation.

Epitope Mapping by Cysteine Mutations—To investigate which cysteines are important for the proper formation of the TB epitopes, each cysteine was mutated to serine in the full-length Pfs48/45 fused to GST (16C). These sixteen individual mutants were expressed in *E. coli* and screened for the formation of conformational epitopes I, IIb, III, and V.

In line with the previously proposed structural organization of Pfs48/45 (4, 18), mutations of any of the six cysteines in the putative C-terminal domain led to the specific loss of epitope I recognition (Fig. 3). This finding corroborated and extended our N-terminal truncation analysis and led to the conclusion that the epitope I mAb recognizes a conformational epitope at the C terminus of Pfs48/45 that requires proper pairing of six cysteines.

Mutations of any of the four cysteines in the putative middle domain affected the recognition of epitopes IIb and III but did not affect I and V. Hence, the middle four-cysteine part of the protein appears to form an independent conformational unit, in agreement with the N-terminal deletion experiments and previously proposed structural organization of Pfs48/45 (4, 18). None of the cysteine mutations affected epitope V (Fig. 3), in line with the fact that this epitope is not sensitive to reducing agents (10).

Refolding and Purification of the Recombinant Proteins—In *E. coli*, the Pfs48/45 protein as well as truncations fused to GST ended up in inclusion bodies. To allow purifications from inclusion bodies, we constructed N-terminal His_6-tagged 16C, 12C, 10C, and 6C proteins. Reduction of the purified proteins before loading on gel and subsequent refolding on the nitrocellulose membrane showed that the 10C, 12C, and 16C (but not 6C) fragments could be partially refolded with respect to epitope I (Fig. 4, *A* and *B*). The proteins were further analyzed for their refolding potential by dilution in renaturation buffer. Two-sided capture ELISA experimentation using mAb III for capture and mAb I and IIb for detection was employed to detect TB epitopes generated upon refolding (Fig. 4*C*). Gametocyte

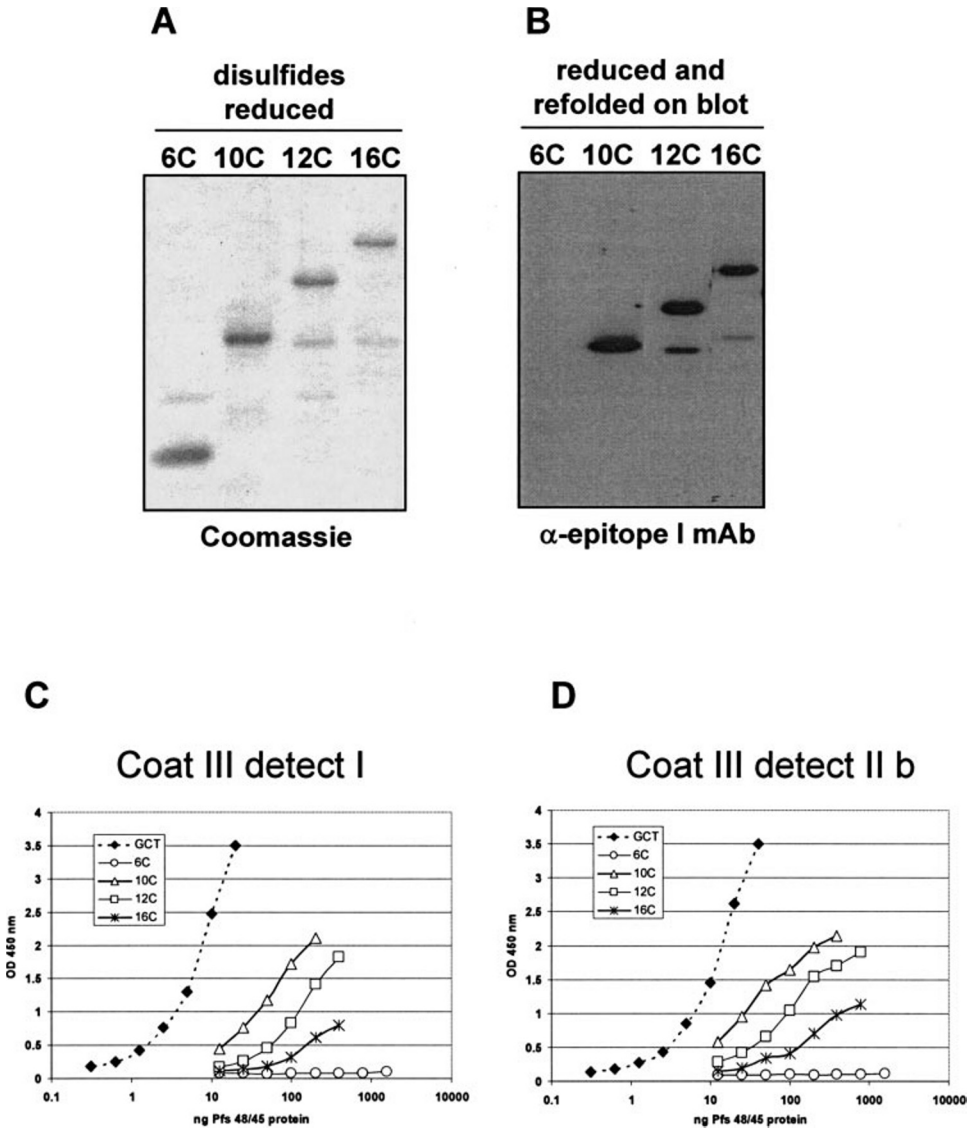

FIGURE 4. **Refolding efficiency of the His-tagged N-terminal truncations.** His-tagged C-terminal Pfs48/45 fragments 6C, 10C, 12C, and 16C were expressed in *E. coli* and purified by Ni-NTA beads. After boiling in sample buffer with 50 mM dithiothreitol, proteins were separated on a SDS-polyacrylamide gel and stained using Coomassie Blue R-250 (*A*) or immunoblot using anti-epitope I (*B*). *C* and *D*, shown are His-tagged 6C, 10C, 12C, and 16C constructs analyzed by two-sided ELISAs using rat mAbs against epitope III for capture and HRP-labeled rat mAbs for detection. On the *y*-axis, the optical density at 450 nm is shown (*OD 450 nm*). The gametocyte extract with known concentrations of the Pfs48/45 protein was used as a positive control. The refolded proteins were treated with 10 mM iodoacetamide before dilution to prevent refolding in the microtiter plate.

extracts with empirically determined concentrations of native Pfs48/45 protein were used as standards. Of the tested recombinant products, the 10C displayed the highest refolding potential with respect to the formation of TB epitopes.

Next, we performed a series of refolding experiments to optimize our refolding conditions. Different conditions such as redox couples, temperature, incubation time, urea concentration, type and amount of detergents, and refolding by dilution and on various types of columns with one-step, gradient, or cyclic renaturation were tested. Single step refolding on a Ni-NTA column yielded the highest refolding efficiency (data not shown). The refolding yielded aggregates in addition to a number of distinct monomeric conformers (Fig. 5*A*). Subsequent concentration by diafiltration and purification on a Superdex 75 column separated monomeric conformers from the aggregates (Fig. 5*B*). Although several monomeric conformers were observed under non-reduced SDS-PAGE (Fig. 5*B*), only one band with an apparent molecular weight of 28 kDa reacted with the mAbs. Capture ELISA experiments (Fig. 5*C*) extended the Western blot results, showing that a fraction of the refolded 10C material displayed proper TB epitopes. Approximately 10–20% of the protein was correctly folded, *i.e.* displayed TB epitopes as estimated from the ELISAs, Western blots, and the silver-stained SDS-PAGE.

Immunogenicity of the Recombinant 10C Preparation—The immunogenicity of the refolded and partially purified 10C protein preparation was assessed by immunization of BALB/c mice. Groups of mice immunized with 10^5 or 10^6 *P. falciparum* (NF54) gametocytes or buffer (PBS) and adjuvant alone served as controls. Mice immunized with the 10C (but not the controls) induced antibodies that detected two bands in gametocyte extracts with the expected mobility of 48 and 45 kDa on Western blots (data not shown). The immune responses were furthermore assessed by gametocyte ELISAs against total gametocyte antigens (Fig. 6*A*) and Pfs48/45 ELISA in which native Pfs48/45 was captured by a mAb (Fig. 6*B*). When analyzed in the gametocyte ELISA, the 10C fragment induced titers of 1/11,400 (S.D. = 1/8,355), which was approximately four times lower as compared with mice immunized with 10^6 gametocytes 1/40,000 (S.D. = 1/14,142) and in a range similar to that of mice immunized with 10^5 gametocytes 1/7,500 (S.D. = 1/2,179) (Fig. 6*A*). Pfs48/45 ELISA revealed antibodies specific for the Pfs48/45 protein with a mean optical density for the five mice of 0.958 (S.D. = 0.447) (Fig. 6*B*).

The ability of the antiserum to recognize native gametocyte or macrogamete protein was further assessed by IFA against whole dried sexual stage parasites and surface immunofluorescence assay (SIFA) using live intact macrogametes/zygotes. Four of five mice sera (10C1, 10C2, 10C4, and 10C5) showed good reactivity to the gametocytes and macrogametes/zygotes but not against asexual stages or Pfs48/45 knock-out parasites.

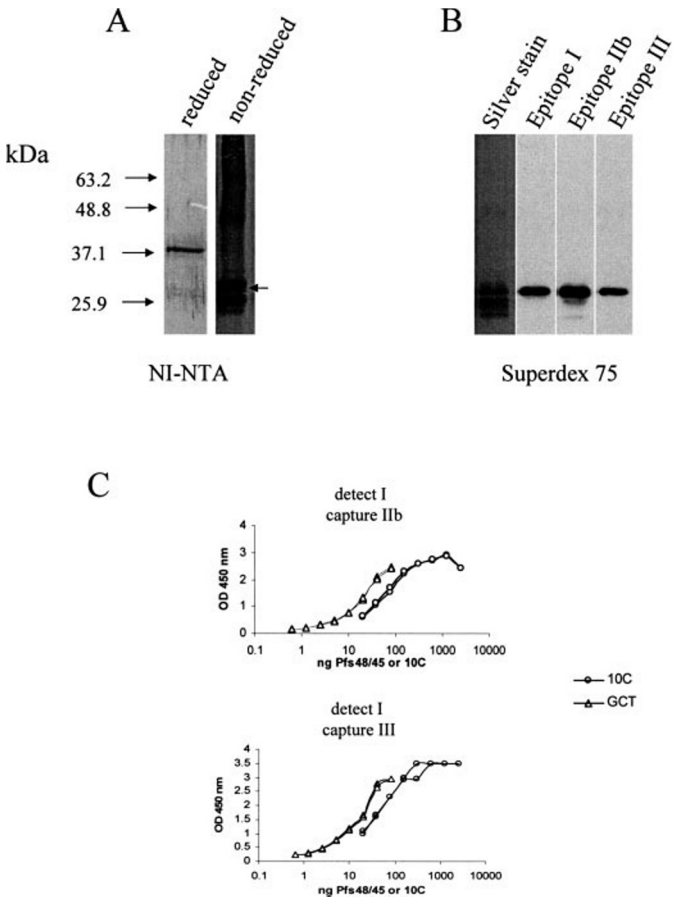

FIGURE 5. **Recognition by TB mAbs of the refolded 10C fragment.** *A*, reduced (*left panel*) and non-reduced (*right panel*) silver-stained SDS-PAGE of the Pfs48/45 10C fragment after refolding on a Ni-NTA column. The *arrow* indicates the position of the conformer with correct conformation. *B*, non-reduced silver stain and Western blot analysis of the 10C fragment after the Superdex 75 purification step analyzed with anti-epitopes I, IIb, and III mAbs. *C*, two-sided ELISA experiment with rat mAbs against epitope IIb (*upper panel*) or III (*lower panel*) for capture of the antigen- and peroxidase-labeled mAb against epitope I for detection. Each dilution point is repeated twice in parallel. The amount of the Pfs48/45 present in the gametocytes was previously determined by titration on a reduced Western blot using polyclonal sera K96 and recombinant Pfs48/45 produced in *E. coli* as a reference.

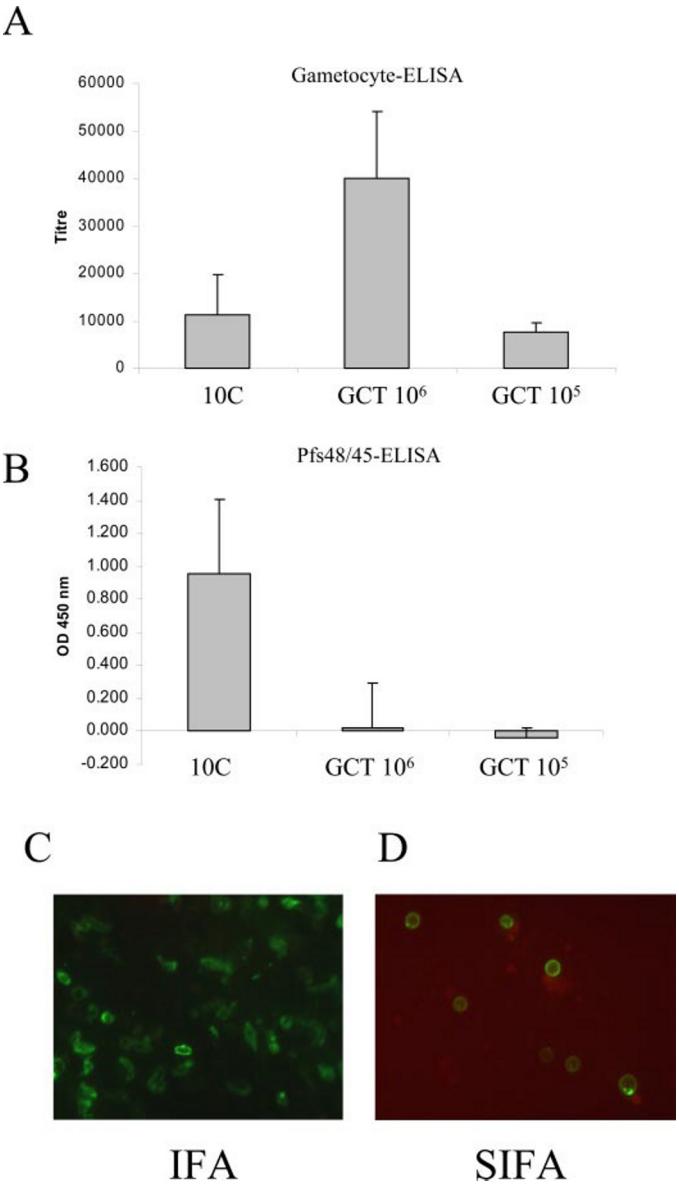

FIGURE 6. **Immunogenicity of the 10C fragment.** *A*, gametocyte ELISA antibody reactivity to total gametocyte extract of sera from mice immunized with recombinant 10C or gametocytes (*GCT*). Data are presented as a mean titer ± S.D. of the five mice sera. *B*, antibody reactivity to Pfs48/45 captured by rat mAb 85RF45.3 in sera from mice immunized with the 10C or gametocytes. Data are presented as mean optical density ± S.D. of the five mice at a dilution of 1/200 of the serum. Shown are immunofluorescence microscopy on *P. falciparum* gametocytes air-dried on a multispot slide (*IFA*) (*C*) and on live intact macrogametes/zygotes (*SIFA*) with anti-10C mouse sera (*D*).

The reactivity of the 10C1 serum is shown in Fig. 6*C*. Sera from mice immunized with buffer and adjuvant did not show a detectable signal (not shown).

Having established the presence of antibodies specific against the native Pfs48/45 in the 10C-immunized mice, we investigated whether the antibodies target known TB epitopes (I and III). Fig. 7 shows a competition of the mice sera against a constant amount of peroxidase-conjugated rat anti-epitope I and III mAbs. The same (but not conjugated) rat anti-epitopes I and III mAbs were used as positive controls and for quantification. All five sera from the 10C immunization competed with the conjugated epitope I mAb, whereas the control sera of adjuvant-immunized mice as well as the preimmune (S0) sera did not (Fig. 7*A*).

Estimations from the amount of competitions in the mice sera compared with the competition observed with the non-conjugated rat mAb revealed that 10C1 serum contained anti-epitope I antibodies at concentrations of ~20 μg/ml. Sera 10C2, 10C3, and 10C5 contained lower titers of anti-epitope I antibodies of ~6 μg/ml. The lowest epitope I competitor 10C4 contained anti-epitope I antibodies in the range of 1–2 μg/ml. Competition with a non-TB epitope III mAb was observed for two of the 10C-immunized mice (Fig. 7*B*). For the 10C1 serum, anti-epitope III antibodies were estimated to be ~100 μg/ml and for the 10C4 ~20 μg/ml.

Previous characterizations of the anti-epitope I mAb revealed that a concentration of 12 μg/ml or more completely blocks transmission (8). Hence, the concentrations of the anti-epitope I antibodies in the 10C1 serum was in the range expected to yield TB effects. The sera 10C2, 10C3, and 10C5 were expected to yield transmission-reducing effects.

Transmission-blocking Activity Induced by the 10C Fragment—Antisera obtained from mice immunized with the recombinant

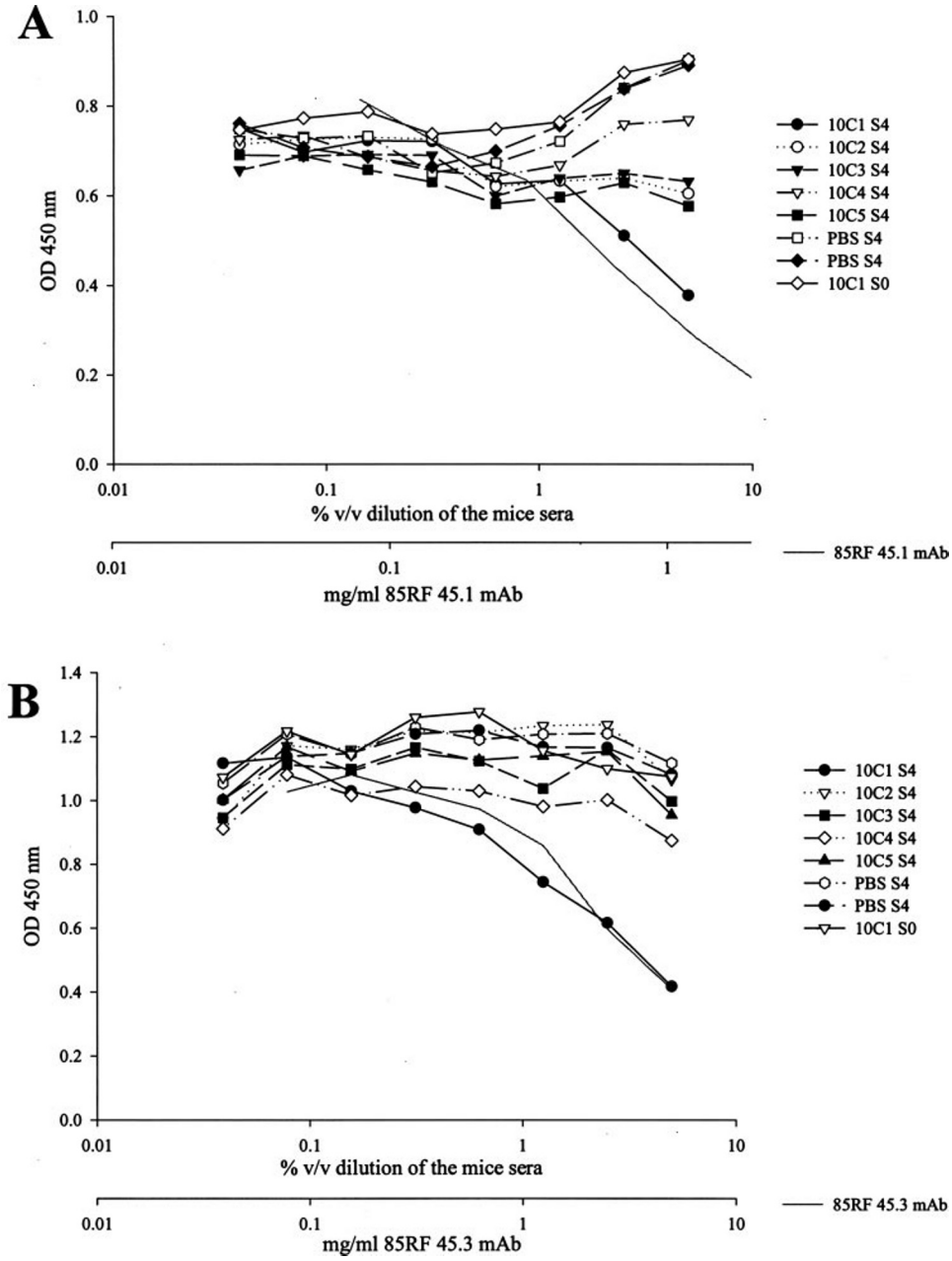

FIGURE 7. **Two-sided competition ELISAs.** mAb against epitope II (85F45.2b) was used to capture a Pfs48/45 molecule from the gametocyte extract. The competition of mice sera immunized with the 10C protein (*10C1 S4*, *10C2 S4*, *10C3 S4*, *10C4 S4*, *10C5 S4*), buffer (*PBS S4*), or preimmune 10C1 S0 was titrated against a constant amount of peroxidase-labeled mAb anti-epitope I (85RF45.1) (*A*) or anti-epitope III (85RF45.3) (*B*). Serum dilutions (from 1/20–1/2560 (5% to 0.039% v/v)) are indicated. Unlabeled 85RF45.1 (*A*) or 85RF45.3 (*B*) was used as indicated on the secondary *x*-axis concentrations as standard references.

10C fragment were analyzed for their TB activities relative to the anti-epitope I competition ELISA. As shown in Fig. 8, the strongest anti-epitope I competitor (10C1) blocked almost completely the oocyst development inside the mosquito midgut by reducing the mean oocyst number from ~7 in the controls to 0.45. Sera with intermediate anti-epitope I antibodies (10C2, 10C3, and 10C5) reduced the oocyst numbers by ~50%. As expected, the concentration of anti-epitope I antibodies correlated to the TB reactivity. Although achieving consistent and high titers of TB antibodies will be required for future vaccine development, the data presented here show that TB antibodies against the native Pfs48/45 can be induced in mice using a recombinant, refolded Pfs48/45 protein fragment.

DISCUSSION

Our study provides the first biochemical and immunological characterization of Pfs48/45 and demonstrates that an N-terminally truncated Pfs48/45 protein, coined 10C, possesses the highest refolding capacity and elicits TB antibodies that can block the fertilization of the *P. falciparum* gametes in a concentration-dependent manner.

A multimodule organization of the Pf48/45 protein emerged as a result of extensive analysis using conformationally dependent antibodies against Pfs48/45. A C-terminal module containing a six-cysteine module was recognized by mAb against epitope I, a middle module containing a four-cysteine module was recognized by mAbs against epitopes IIb and III, and an N-terminal module also containing a six-cysteine motif was recognized by the epitope V mAb. The cysteines in the central and the C-terminal modules appear to be crucial for proper presentation of the TB epitopes I, IIb, and III. Proper display of the TB-blocking epitope V in the N-terminal module did not depend on cysteines nor was it sensitive to treatment with reducing agents (10). Limited trypsin digestion and refolding experiments indicated that the middle four-cysteine and the C-terminal six-cysteine module are likely to interact to form one stable domain of 10 cysteines, whereas the N-terminal module of Pfs48/45 appears to form a separate domain.

Similarly, related four- and six-cysteine modules are present in ten other unique-to-*Plasmodium* proteins (4, 18). Taken together, our study corroborates and extends previous *in silico* predictions on the disulfide bond structures and domain organization of Pfs48/45 and related proteins (4, 18). Although most of the Pfs48/45-related family members are largely uncharacterized, some of them are obvious TB vaccine candidates. Pfs48/45, P36p (5, 19), and Pfs230 (20) are expressed on the surface of the parasite and have an essential function in development. In addition, Pfs230 and Pfs48/45 are direct targets for TB antibodies (11, 20, 21). Although recombinant expression of

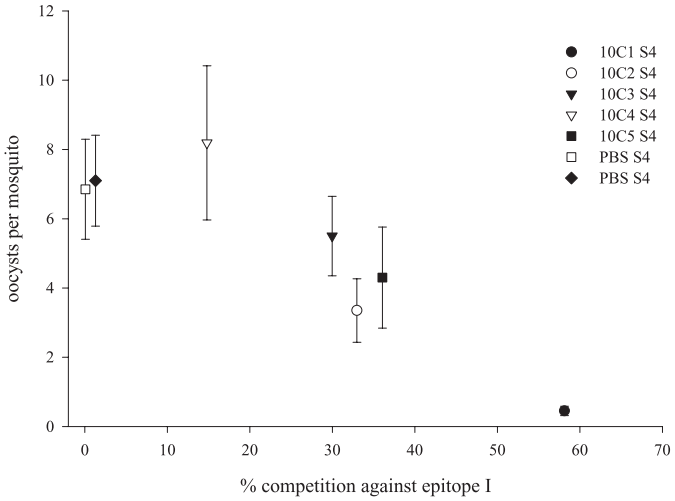

FIGURE 8. **Transmission blocking activity correlates to the concentration of the anti-epitope I antibodies.** Mean oocyst numbers/mosquito ($n = 20$) are related to the concentration of the anti-epitope I antibodies in the sera from mice immunized with the 10C protein (*10C1 S4*, *10C2 S4*, *10C3 S4*, *10C4 S4*, *10C5 S4*), or PBS buffer (*PBS S4*) as measured by competition ELISA and displayed as a percentage of competition against epitope I mAb.

these proteins in a proper conformation is expected to be cumbersome, combinations of various four- and six-cysteine modules may yield a successful production strategy. Cloning and expression of protein fragments similar to 10C (four-cysteine module adjacent to six-cysteine module) from Pfs230 or other related proteins is expected to reveal the feasibility of this approach.

Previous studies have revealed that, in particular, epitope I has strong TB potential (8). This property seems not related to the origin of mAb itself but specific for the epitope that it recognizes, as all of the types of mAbs against epitope I (mouse and rat) block transmission completely, whereas mAbs against epitopes IIb and III have no effect on their own (8). The C-terminal six-cysteine module turned out to be the minimal portion of Pfs48/45 that could display epitope I (Fig. 4). It appeared, however, that the fragment was not stable upon storage and highly sensitive to reducing conditions (data not shown). Interestingly, we consistently observed that the C-terminal six-cysteine module readily displayed epitope I on immunoblot when expressed as a GST fusion (Fig. 2) but not with the hexahistidine tag (Fig. 4). Apparently, the GST moiety has a stabilizing effect on the conformation of Pfs48/45, similar to the middle four-cysteine module itself. The 10C fragment was stable upon storage and more resistant to treatment with reducing agents than the C-terminal 6C fragment (data not shown). Superdex 75-enriched 10C conformer can be stored for at least 8 weeks at 4 °C without detectable loss of reactivity with TB mAbs.

The ability of the refolded and partially purified 10C fragment to induce TB antibodies in rodent models such as mice was subsequently demonstrated in immunization experiments. The protein is immunogenic, as it induced antibodies that specifically recognized the native Pfs48/45 protein. Competition ELISAs revealed that Pfs48/45 antibodies directed against TB blocking epitope I are present in the sera of mice immunized with the 10C protein. The concentration of epitopes I and III antibodies varied in the five mice; one 10C1 was in the required range to efficiently block transmission (20 μg/ml). The same sera contained anti-epitope III antibodies at a concentration of 100 μg/ml, indicating that epitope III is immunodominant over epitope I, which is in accordance with the observation that sera from malaria-exposed individuals contain predominantly non-TB anti-epitope III antibodies and relatively low levels of anti-epitope I antibodies (11). Apparently, the middle four-cysteine module, recognized by epitope III, retains higher immunogenicity as compared with the six-cysteine module, recognized by epitope I. The six-cysteine module from the other hand is crucial for the protective efficacy.

The potency of malaria TB vaccines is, among others, dependent on the concentration of the induced antibodies and the quantity of the fertilization-competent gametocytes (8, 22, 23). Thus, the required magnitude of antibody responses is expected to depend on the load of mature gametocytes. Field data indicate that low concentrations of "submicroscopic" (<5000 gametocytes/ml) gametocytemia constitute a considerable proportion (75%) of the human infectious reservoir in *e.g.* Kenyan children (24). Mosquito infection rates for these submicroscopic carriers are ~4.1%; for the other carriers with microscopic densities, the infections rates are 8.3%. In our transmission blocking assays, mosquitoes were fed on *in vitro* grown gametocyte cultures that contained 1.5×10^6/ml mature gametocytes with infection rates of >90% of the fed mosquitoes. Therefore, it is expected that, under field conditions, the 10C-induced antibody responses may yield sufficient TB effects.

The variability in the amount of induced antibody titers in the five 10C-immunized mice resulted in variability in outcome on the TB assay. For the future, it will be necessary to achieve uniform, sustained, and high titer anti-epitope I antibody responses in all of the 10C-immunized animals or human individuals. For Pfs25H, another malaria TB vaccine candidate, the chemical conjugation to the outer membrane protein complex of the *Neisseria meningitidis* serogroup B and adsorption to aluminum hydroxyphosphate induced high and persistent antibody levels that yielded the desired uniform TB activity (25). This or similar approaches, as described in Ref. 26 or 27, will be useful in the future in Pfs48/45-based vaccine formulations, as they are expected to yield higher and more uniform titers of TB antibodies and guarantee the efficacy of the 10C vaccine.

In conclusion, the induction of TB antibodies using recombinant Pfs48/45 subunit protein provided a proof-of-concept for a recombinant Pfs48/45-based TB vaccine. Future efforts will be directed toward achieving a more homogeneous and correctly folded 10C preparation as well as improving the immunization schemes and vaccine formulations.

Acknowledgments—We thank Geert-Jan van Gemert for culturing the parasites and Wynand Eling for helpful discussions.

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